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PRINCIPAL INVESTIGATOR: Leah Novinger
Leah Novinger, PhD

CONTRACTING ORGANIZATION: University of Vermont & State Agricultural College
Burlington, VT 05405

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| 14. ABSTRACT The purpose of this project is to identify breast cancer antigens and autologous antibodies that breast cancer patients make to those antigens. The scope of this project is the development of new targeted therapeutics to treat breast cancer. The major findings of this project are the methods developed for successful extraction of mRNA from breast tumor specimens and synthesis of a cDNA library that can be expressed as protein fragments on the surface of phage. Future plans include identifying tumor associated antigens via binding of protein fragments to antibodies found in patient serum and characterization of the antibodies identified. The identification of new breast cancer antigens and characterization and replication of autologous antibodies in the laboratory using phage display represent significant and novel information for the diagnosis and treatment of breast cancer. | | | | | | |
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Introduction

The purpose of this research is to identify breast cancer specific protein fragments from a patient and identify autologous antibodies that bind to those fragments. Phage display methods will be used on tumors from patients with breast cancer as they are a useful tool for identifying cell surface markers (by creating a cDNA library of tumor cells)[1] or for production of antibodies for delivering chemotherapy or radiation [cite]. The scope of this project is to develop a method to utilize a patient's own immune system to make targeted therapeutics. In addition, the project will identify cell surface markers for breast cancer cells that have potential use in breast cancer diagnosis.

Body

An appended presentation is included in this document that was created for my thesis committee meeting in September. It included detailed descriptions of methodology in the figure legends.

Phase 1. Generation of breast cancer phage displayed library and identification of autologous antibodies to phage displayed breast cancer proteins.

Task 1. Create cDNA phage library from a patient with breast cancer. We were able to obtain a de-identified breast tumor specimen, isolate mRNA from it, and create a cDNA library. This process took longer than anticipated due to the difficulty of obtaining optimal quality mRNA from tumor fragments, although we were able to develop sufficient methods for obtaining tumor specimens in the hospital and bringing them back to the lab for homogenization and RNA extraction (see slides 19 - 26 in the attached presentation). During this process, we further developed methods for cDNA synthesis using breast cancer cell line RNA (slides 29 - 33 in the attached presentation). We have successfully created a cDNA library using a de-identified breast tumor specimen (sample labeled 188-6, slide 29 attached presentation).

However, we have been unable to express the library using phage display to date, most likely due to lower yields than expected during cDNA synthesis reactions. A new PCR based method is currently being developed for synthesizing higher quantities of cDNA using the same or smaller quantities of RNA.

Anticipated Outcome: a library of protein fragments from the primary breast cancer displayed on phage

Task 2. Select phage displayed breast cancer antigens that bind to immobilized antibodies from the same breast cancer patient's serum. Due to the delay in the development of methods for cDNA library synthesis, task 2 was not accomplished in the past year. Recently, we have considered utilizing pre-made peptides to common SEREX antigens to develop methods for this task while the methods from task 1 are being optimized. Anticipated Outcome: isolation and identification of breast cancer protein fragments (deliverable) to which the patient has pre-existing antibodies.

The tasks in phase 2 of the statement of work have not been completed yet.

Phase 2. *Ex vivo* identification and amplification of patient antibodies that bind to breast cancer protein fragments

Task 3. (16 months) Generate phage display antibody library from the patient's B-cells

Task 4. (20 months) Select phage displayed antibodies that bind to selected breast cancer proteins

Task 5. (34 months) Determine sensitivity and specificity of individual phage antibody clones for binding to breast cancer.

Key Research Accomplishments

- Sufficient quality mRNA extractions were obtained from tumor tissue and cultured cells.
- cDNA libraries synthesized are structurally intact and ready for vector insertion, however, variations on synthesis reaction will be explored to increase cDNA yield.
- Control experiments for vector ligation and insertion demonstrate optimal method development in anticipation of completion of cDNA libraries.

Reportable Outcomes

Presentations

"Construction of a Protein Expression Library From Human Breast Cancer Tissue" UVM MD/PhD Seminar. November 13, 2008

"Construction and Applications of a Protein Expression Library from Human Breast Cancer Tissue" UVM Cell and Molecular Biology Program Seminar. December 2, 2008

"Construction and Application of a Protein Expression Library from Human Breast Cancer Tissue" UVM Graduate Research Day. April 16, 2009

"Creation of a Phage Displayed Breast Cancer cDNA Library" UVM MD/PhD Research Day. July 17, 2009

Posters

Novinger, L. Creation of a Phage Displayed Breast Cancer cDNA Library. UVM Cell and Molecular Biology Retreat. August 19, 2009

Conclusions

The results presented in this summary have revealed the difficulty in creating a cDNA library that can be expressed on the surface of phage. Intact RNA is more difficult to obtain from patient tumors than from cell culture, which has historically been the source of phage displayed cDNA libraries [cite]. Currently, methods are being developed for consistent creation of cDNA libraries that can be expressed on the surface of lambda phage.

These libraries will have a robust capacity for the evaluation of tumor associated antigens and their corresponding autoantibodies in a breast cancer patient using the SEREX technique [2]. The autoantibodies identified can be characterized and replicated in the lab for use of targeted therapeutics or monitored as biomarkers or for diagnostic purposes [3]. Furthermore, the differential repertoire of antigens expressed on the surface of the patient's tumor is also very useful information. Identifying the over expressed or aberrant antigens particular to the patient is the primary reason for making a phage displayed library of tumor cDNA fragments [4].

On the other hand, in the interest of time and feasibility, some flexibility may be necessary and the most common SEREX antigens may be substituted for the cDNA library as protein targets and panned with the patient antibody library while cDNA synthesis methods are modified [5].

References

1. Sergeeva, A., et al., *Display technologies: application for the discovery of drug and gene delivery agents*. Adv Drug Deliv Rev, 2006. **58**(15): p. 1622-54.
2. Sahin, U., et al., *Human neoplasms elicit multiple specific immune responses in the autologous host*. Proc Natl Acad Sci U S A, 1995. **92**(25): p. 11810-3.
3. Pavoni, E., et al., *Tumor-infiltrating B lymphocytes as an efficient source of highly specific immunoglobulins recognizing tumor cells*. BMC Biotechnol, 2007. **7**: p. 70.
4. Minenkova, O., et al., *Identification of tumor-associated antigens by screening phage-displayed human cDNA libraries with sera from tumor patients*. Int J Cancer, 2003. **106**(4): p. 534-44.
5. Pavoni, E., et al., *A study of the humoral immune response of breast cancer patients to a panel of human tumor antigens identified by phage display*. Cancer Detect Prev, 2006. **30**(3): p. 248-56.

Appendices

Please see the attached presentation.

| | |
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| <p>Thesis Committee Presentation</p> <p>Leah Novinger September 9, 2009</p> | <p>Outline</p> <ul style="list-style-type: none">• Coursework• Background• Project Goals and Hypothesis• Completed Work• Next Year's Work• Conclusions |
|--|--|

Coursework and Requirements

- Required Coursework
- Research Credits
- Other Requirements

3

Background Overview

- Targeted Therapeutics
- Autoantibodies
- SEREX



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Targeted Therapeutics

- Targeted therapies for breast cancer patients can reduce resistance to treatments
 - Resistance occurs eventually in most patients due to genetic amplification (Gonzales-Angulo 2007)
- Criteria for Antibody Use
 - Affinity
 - Specificity
 - Bioactivity
- Target Validation

Patient 1

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Tumor Immunology

- A beneficial immune response to cancer cells exists, but may not be enough to eliminate the tumor
 - "The presence of tumor infiltrating lymphocytes is associated with better prognosis in individual patients" (Jäger 2007)

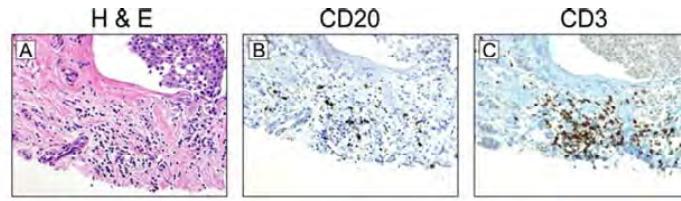


Image source: Coronella et al. J Immunol. 2002 Aug 15;169(4):1829-36.

Tumor Immunology

- A beneficial immune response to cancer cells exists, but may not be enough to eliminate the tumor
 - "The presence of tumor infiltrating lymphocytes is associated with better prognosis in individual patients" (Jäger 2007)
- Tumor response is associated with chronic inflammation. By converting to an acute inflammatory response, we can better eliminate tumors
- The antibody response may be associated with different subsets of disease
 - ER/PR + patients have more autoantibody reactivity than ER/PR - pts

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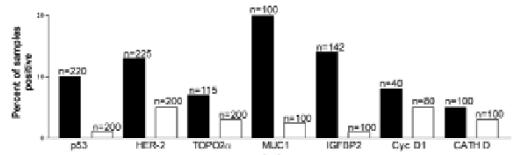
Autoantibodies as biomarkers

- HER-2/neu Ab correlate with protein expression tumor cells and specificity to cancer cells (Lu 2008 and Disis 1997) but are 20 fold lower than therapeutic concentrations (Coronella-Wood, 2003)
- Tumor autoantibodies can be detected in sera before cancer diagnosis (Lubin 1995, Li 2005), and roughly half of breast cancer patients have an antibody response to the primary tumor (Cornella-Wood, 2003)
 - Best responses are associated with early disease

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Autoantibodies as Biomarkers



Antibodies to tumor associated antigens are more frequently detected in sera from breast cancer patients than from normal donors. Shown are the percentages of individuals positive for serum antibody to 7 tumor antigens. Gray columns show the response in patients; white columns show the response in control normal donors. The number of patients or controls tested for each antigen were indicated at the top of the column. The antibody responses to TOPO2α, IGF1P2, cathepsin D (CATH D), MUC1, and cyclin D1 were measured using recombinant ELISA. The antibody responses to p53 and HER2 were measured using capture ELISA as previously described.(40) A sample was defined as positive if the antibody concentration was greater than the mean \pm 3SD of the reference population.

Published in: Hailing Lu; Vivian Goodell; Mary L. Disis; *J. Proteome Res.* 2008, 7, 1388-1394.
DOI: 10.1021/pr700818f
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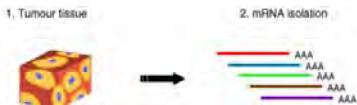
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IRB Approval and Clinical Side of Project

- IRB Approval obtained for ten breast cancer patients and for normal tissue
- PRC Approval obtained from the Vermont Cancer Center
- Current work is with cell lines and banked tissue
- Work with consented patients will start soon

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Modified SEREX



Adapted From *Cancer Lett.* 2007 Apr 28;249(1):110-9

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Establishing Protocol: Cell Line RNA Isolation

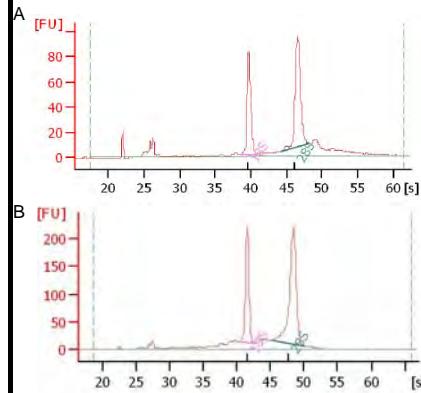


Figure 1. SkBr3 cells were cultured in T75 flasks to 80-90% confluence. Cells were then trypsinized and spun down at 1000g for 5 minutes. Cells were resuspended in 1ml Trizol/10⁶ cells. Total RNA was isolated with ethanol precipitation and a QIAGEN column. Figures A and B are representative of typical samples. Analysis demonstrates two rRNA peaks with little degradation of samples. RIN numbers typically range from 8.5-10.

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Obtaining and Preparing Tissue: Early Homogenization Expts

Causes of poor RNA Quality

- Necrotic tumor

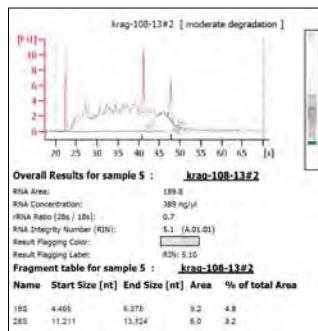
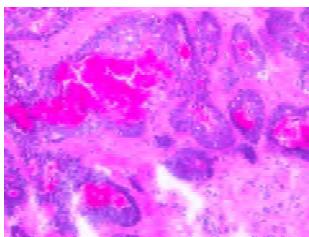


Figure 2. Metastatic colon cancer tissue was obtained from surgical pathology and flash frozen in OCT. Tissue was analyzed via H and E stain (A) and then remaining sample was used for RNA Isolation (B). Methods described in Figure 1 were used. Analysis demonstrated moderately degraded RNA.

Obtaining and Preparing Tissue: Acquiring Tissue Samples

Causes of poor RNA Quality

- Necrotic tumor
- Delays in obtaining tissue

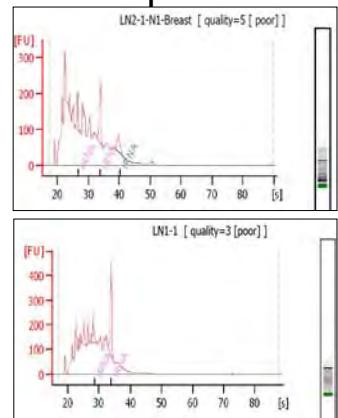


Figure 3. Tissue samples were obtained from FAHC Surgical Pathology after being stored at 4C for several hours. RNA was isolated using the same method described in Figure 1. Analysis demonstrated severely degraded total RNA.

Homogenization Methods

Homogenization methods

- FastPrep Instrument
 - Lysing Matrix

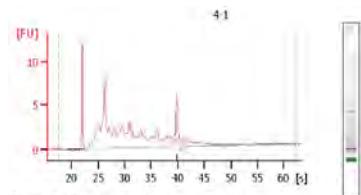


Figure 4. Tissue was obtained from surgical pathology, flash frozen, and stored at -80C. On the day of RNA isolation, the tissue sample was briefly thawed, cut up with a sterile razor blade, and put in 1ml Trizol with Lysing Matrix. The sample was put in a FastPrep Instrument for 4-5 rounds of 25 secs each, and stored on ice between rounds. RNA was then isolated in the manner described in Figure 1. Results show severely degraded RNA.

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Later Methods

Changes in Protocol

- Snap freezing samples shortly after excision
- Thorough homogenization with Polytron

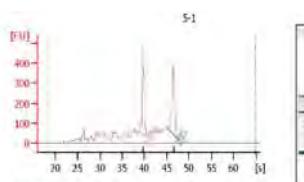
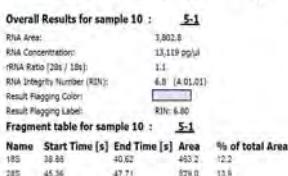


Figure 5. Tissue samples were obtained from Surgical Pathology, flash frozen and stored at -80C. On the day of RNA isolation, the sample was removed from -80C, transferred to a 50ml conical tube with several ml of Trizol. The sample was subjected to a polytron homogenizer for 5-10 seconds and then put on ice. RNA was then isolated in the manner described in Figure 1. Results show mildly degraded RNA.



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Tumor Tissue RNA Isolation

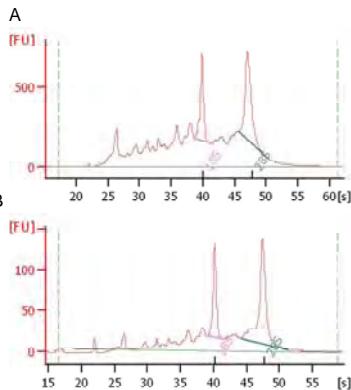


Figure 6. Deidentified breast carcinoma tissue was obtained from FAHC Surgical Pathology and flash frozen. Samples were stored at -80C. On the day of the experiment, samples were removed from the freezer and transferred to a conical tube with several ml of Trizol and homogenized with a polytron homogenizer. Following RNA isolation, samples were evaluated with an Agilent Bioanalyzer. Results in A and B are representative samples and demonstrate two rRNA peaks with slightly more degradation than seen with SkBr3 cells.

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Lymph Node RNA Isolation

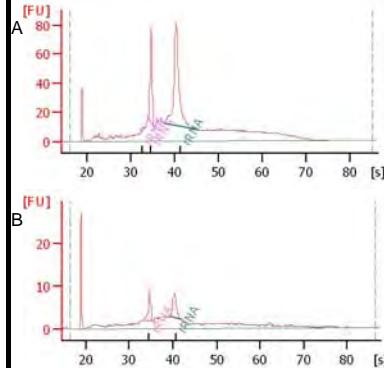


Figure 7. Representative total RNA isolated from de-identified breast cancer sentinel lymph node scrapings. Both scrapings came from the same patient and were stored in Hanks BSS with RNase Inhibitor and kept on ice until Trizol extraction. Figure A shows relatively intact RNA while Figure B, collected on the same day, shows degraded RNA.

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Interim Conclusions

- IRB and PRC Approval was obtained for this project
- Obtaining tissues
 - Fresh samples of non-necrotic tumor
 - Snap Freeze in FAHC Surgical Pathology
- RNA Isolation
 - FastPrep was inefficient for size and type of tissue
 - Polytron provides best homogenization of samples

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Cell Line mRNA Isolation

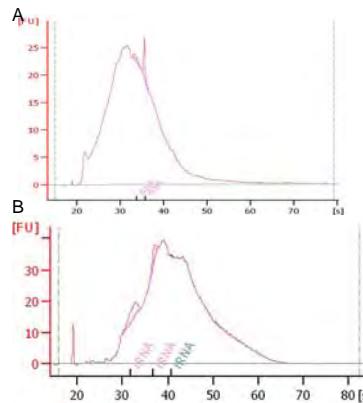
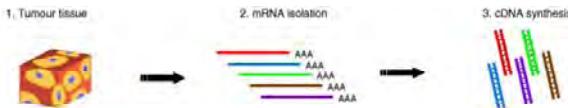


Figure 8. mRNA was isolated from the SkBR3 cell line total RNA. In both A and B, analysis with Agilent Bioanalyzer demonstrates a bell shaped curve indicative of RNA strands of varying length. Slight contamination of with rRNA can be observed in both samples.

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Modified SEREX



1. Tumour tissue

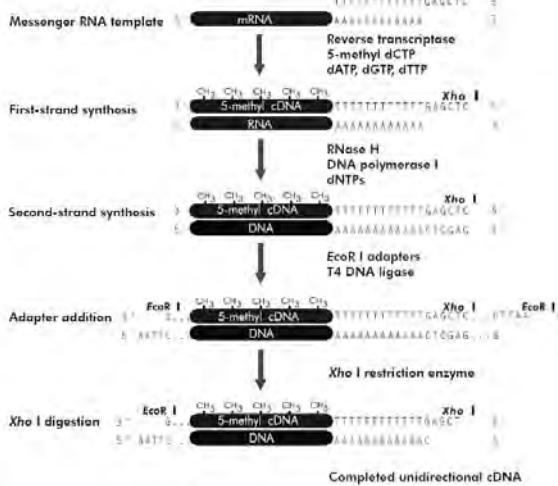
2. mRNA isolation

3. cDNA synthesis

Adapted From Cancer Lett. 2007 Apr 28;249(1):110-9

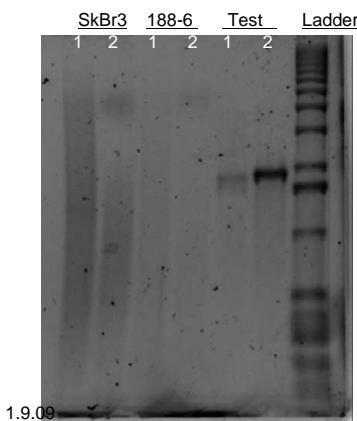
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Oligo(dT) linker-primer



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cDNA Library Synthesis



1.9.09

Figure 9. cDNA was synthesized from 2.5ug mRNA from SkBr3 cells, breast carcinoma tissue (188-6) or Test RNA (all 1.6kB) provided by Stratagene. First and second strand synthesis reactions were evaluated by alkaline agarose electrophoresis, which denatures bound strands but retains secondary structure that may have inhibited synthesis reactions. A ladder ranging from 1-10kB was used in the experiment. A dark smear was observed for both first and second strand SkBr3 synthesis reaction. A lighter but similar looking smear was observed for 188-6 samples (de-identified breast cancer tissue). An isolated band was observed for Test RNA, consistent with expected results since all mRNA was the same length. No high or low molecular weight bands were observed, which would be indicative of "hairpinning", or folding of the RNA or cDNA that would have prevented a proper synthesis reaction from occurring.

Fractionation Experiments

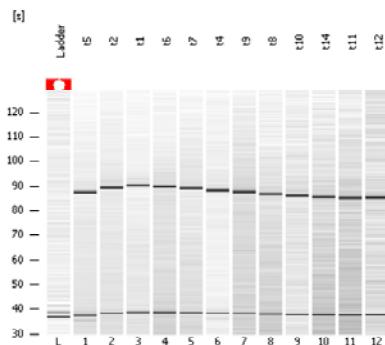
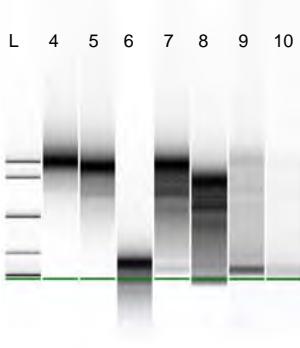


Figure 10. cDNA was synthesized from Stratagene test RNA and fractionated on a Sepharose column. Bioanalysis results demonstrate lower quantities of RNA than determined via Nanodrop and an unknown high molecular band exists, presumably due to column dye.

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Fractionation Experiments



Ladder runs from 0.5kB to 2kB

Figure 11. The SkBr3 cDNA library was added to an Invitrogen S-500 HR resin column and 20 fractions were collected. Spectrometric analysis suggested that Fractions 4 -10 contained enough cDNA for quality evaluation. Using Agilent Bioanalysis, it was determined that Fractions 4, 5, 7, 9, and 10 contained cDNA no less than 0.5kB long. Unexpected results occurred with Fractions 6 and 8, which contained some small molecular weight cDNAs less than 500bp long.

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cDNA Library Synthesis (2)

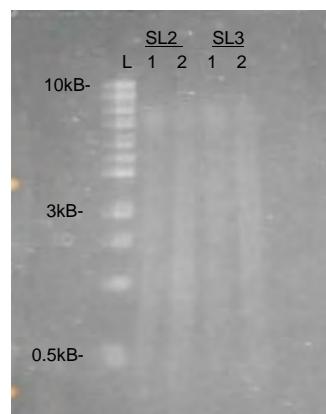
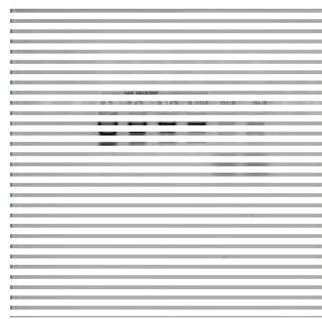


Figure 12. More SkBr3 RNA was used to create two more cDNA libraries. Synthesis reactions produced intact cDNA of the correct size, however, only small amounts of cDNA were synthesized.

Ladder Titration

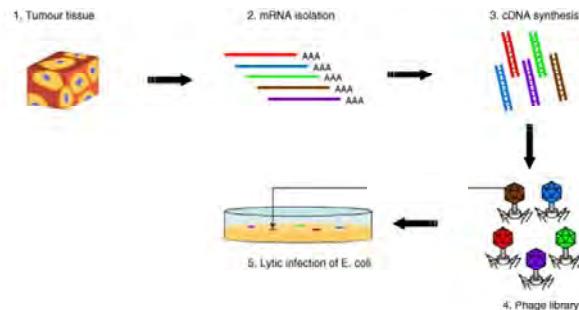
Based on findings both libraries probably contain far less than 500ng of cDNA and synthesis reaction was not complete

- This correlates with early bioanalysis results



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Modified SEREX



Adapted From Cancer Lett. 2007 Apr 28;249(1):110-9

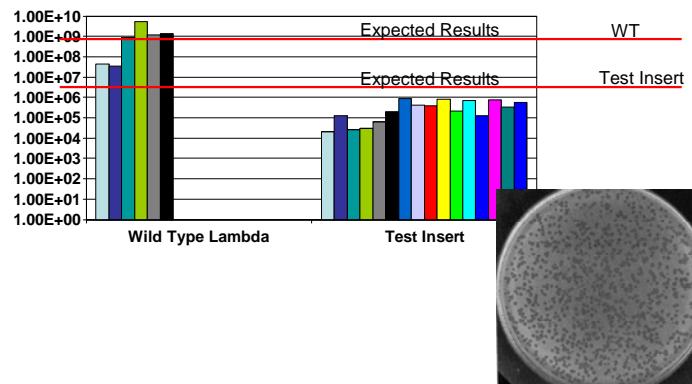
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Ligation and Packaging Experiments

1. Obtained correct titer with wild type phage and packaging extracts
 - Experimental titers < Expected
 - Replaced packaging extracts and obtained experimental titers in the correct range
2. Used test insert to evaluate vector ligation and packaging
 - Experimental titers < expected
 - Suspected bad T4 DNA Ligase
3. Packaged test cDNA library and SkBr3 library

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Measuring Efficacy of Insertion



Interim Conclusions

- RNA Isolation
 - High quality extractions were obtained from SkBr3 cells
 - Sufficient quality extractions were obtained from tumor tissue
 - Isolation of RNA lymph node tissue is difficult and better methods must be developed
- cDNA Synthesis
 - cDNA libraries are structurally intact and ready for vector insertion
 - However, variations on synthesis reaction will be explored to increase cDNA yield
- Size Fractionation:
 - Poor product retention yields inconsistent results occur with current methods
 - Faster methods with higher product retention using PCR Clean Up and molecular weight spin columns are currently being developed for this protocol
- Ligation and Packaging Reactions
 - Still in development pending cDNA synthesis reactions

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Next Year's Work - SEREX

- Continue to work on protocol to synthesize sufficient quantities of cDNA (0 - 3 months)
- Efficiently ligate cDNA strands into a vector and package into lambda phage. (3 – 6 months)
- Pan phage displayed library on breast cancer patient serum and identify protein fragments (6 - 9 months)
 - Bead based assay using a column
 - Binding analysis via ELISA
- Begin generating scFv libraries from patient B cells (9 – 12 months)

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